CELL CYCLE
2021, VOL. 20, NO. 19, 2066–2076
https://doi.org/10.1080/15384101.2021.1970305

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RESEARCH PAPER

# Ubiquitin specific peptidase 33 promotes cell proliferation and reduces apoptosis through regulation of the SP1/PI3K/AKT pathway in retinoblastoma

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**ABSTRACT**

Ubiquitin-specific protease 33 (USP33), a deubiquitinating enzyme (DUB), has been identified to serve as a tumor suppressor or an oncogene in different cancers. However, its role in retinoblastoma (RB) remains unknown. Here, we aimed to uncover USP33 expression profile and function in RB, and disclose the underlying mechanism. USP33 levels in RB tissues and cells were determined using RT-qPCR and western blotting assays. USP33 effects on cell growth, cycle, apoptosis and tumorigenesis were studied using MTT, Edu, cycle and western blotting and *in vivo* assays. The results showed that USP33 expression levels were elevated in RB tissues and cells as compared with normal retinal tissues and cells. Downregulation of USP33 in RB Y79 and WERI-RB1 cells leaded to significant increases in cell apoptosis, G1 phase arrest and tumorigenesis, and reductions in cell growth and G2 and S phase arrest, as well as inhibited the activation of the PI3K/AKT signaling. SP1 overexpression abolished the roles of USP33 downregulation in modulating the activation of PI3K/AKT signaling, cell growth, apoptosis, and cell cycle. This study uncovered that USP33 promoted the progression of RB through regulation of the SP1/PI3K/AKT pathway.

**ARTICLE HISTORY**
Received 25 February 2021
Revised 22 June 2021
Accepted 24 June 2021

**KEYWORDS**
USP33; cell cycle; apoptosis; growth; PI3K/AKT signaling

## Introduction

Retinoblastoma (RB) initiating from the immature retinal cells is a rare malignancy that prevailingly affects young children and accounts for about 4% of cancers happened in children and infants [1]. The incidence of RB is 1/15, 000 ~ 20, 000, which amounts to about 9000 new cases worldwide annually [2]. Patients with RB appear many symptoms, such as leukocoria, deterioration of vision, red and irritated eyes, and growth retardation [3]. Although it is widely accepted that mutations in the RB1 gene are risk factors for this cancer, the mechanisms underlying RB progression are still poorly understood [4]. Additionally, delayed diagnosis and treatment can cause RB exacerbation and migration [5]. Therefore, it is needful to further reveal the mechanism by which PB occurrence and development.

Chakraborty et al. [6] reported in 2007 that genes including PIK3CA, AKT1, FRAP1, and RPS6KB1 are dysregulated in RB tissues, indicating the possible role of the PI3K/AKT pathway in RB progression. Then, more and more studies focused on PI3K/AKT signaling and uncovered an important role of PI3K/AKT signaling in RB development [7,8]. The PI3K/AKT pathway modulates gene translation, which encodes pro-oncogenic proteins, causing enhancements in cancer proliferation, migration, and invasion [9–11]. Targeting the PI3K/AKT signaling may be a potential method to repress RB progression.

Ubiquitin-specific protease 33 (USP33) is a deubiquitinating enzyme (DUB) and belongs to the ubiquitin-specific protease family [12], and serves as a substrate, which combines with von Hippel-Lindau tumor suppressor (VHL) protein E3 ligase [13]. USP33 closely associates with various physiological events, such as mitophagy, hepatocyte growth factor (HGF)-dependent epithelial cell scattering and thyroid hormone activation [14–16]. Noticeably, evidence has demonstrated that USP33 takes part in carcinogenesis, but its roles are different depends on the contents of cancers [17,18]. For instance, USP33 level was increased in hepatocellular carcinoma (HCC), and USP33 repression weakened HCC invasion and migration both *in vitro* and *in vivo*, indicating

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that USP33 acts as an oncogene in HCC [19]. However, USP33 level was decreased in colorectal cancer, which correlated with advanced tumor grade, higher lymph node metastasis rate, and poor survival; upregulation of USP33 inhibited cell migration by deubiquitinating and stabilizing Robo1 [18]. However, USP33 role in RB progression is still needed to be illustrated.

SP1 is a transcription factor and plays an oncogenic role in RB [20,21]. Gan et al. [19] found that USP33 increased SP1 expression in HCC. Yi et al. [22] reported that SP1 can promote the activation of PI3K/AKT/c-Jun signaling in glioma, indicating that USP33 may trigger the activation of PI3K/AKT/c-Jun through SP1.

As a result, the present study was performed to explore USP33 function in RB progression, and to uncover whether the SP1/PI3K/AKT signaling was a target of USP33.

## Materials and methods

### *Clinical tissue samples*

Sixty RB and the adjacent normal tissues in paraffin were obtained from the Third People's Hospital of Changzhou. The age of the patients ranged from 0 to 6 years, with an average age of 2.3 years. The written informed consents were obtained from patients or their parents. Additionally, we got approval for this study from the Ethics Committee of the Third People's Hospital of Changzhou before this study.

## Immunohistochemistry (IHC)

Paraffin sections of RB tumor and the adjacent normal tissues were sliced into sections of 4-mm thickness. Then, the sections were submitted to dewaxing and incubation with 3% $H_2O_2$ at room temperature for 10 min, antigen repairing with Tris-EDTA and sealing with 5% goat serum (diluted in PBS). Next, the sections were incubated overnight with primary antibody against USP33 (1:150 dilution; cat no. ab237510, Abcam, MA, USA), SP1 (1:2000 dilution; cat no. #9389, Cell Signaling Technology, MA, USA), p-AKT (1:100 dilution; cat no. #4060, Cell Signaling Technology) or c-Jun (1:400 dilution; cat no. #9165, Cell Signaling Technology) at 4°C, and then incubated with secondary antibody. Chromogen 3, 3′-diaminobenzidine tetrachloride (DAB) (Serva, Heidelberg, Germany) was used as a substrate. The cell nucleus was dyed with Harri's hematoxylin solution.

## Cell lines

Two human RB cell lines, including Y79 and WERI-RB1, and retinal pigmented epithelium ARPE-19 were purchased from American Type Culture Collection (ATCC, VA, USA) and cultured in RPMI-1640 medium, supplemented with 10% FBS (Fatal Bovine Serum) and 1% penicillin and streptomycin. The cells were placed at 37°C in an atmosphere containing 5% $CO_2$. RPMI-1640 medium and FBS were purchased from Thermo Fisher Scientific (USA).

## Upregulation and downregulation of USP33/SP1 in human RB cells

The coding sequence of the human USP33/SP1 gene was amplified and inserted into the pcDNA3.1 plasmid, with empty plasmid as a negative control. Then, cells were transfected with USP33/SP1 plasmid and control vector using Lipofectamin 2000 based on the manufacturer's instructions.

The shRNAs named shUSP33#1 and shUSP33#2 purchased from GenePharma (Shanghai, China) were introduced into cells using polybrene (5 μg/ml) to downregulate USP33 level in RB cells. Then, the infected cells were cultured with RPMI-1640 medium containing G418 (geneticin) for 14 days to produce the stable transfected cell lines. The transfection efficiency was determined using western blotting.

## Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from RB tissues and cells using TRIzol reagent (Invitrogen, USA), and then subjected to cDNA synthesis with the help of PrimeScript RT Master Mix kit (RR036A; Takara). Next, the cDNA was applied to PCRs detection with 2× SYBR Green PCR Mastermix

(Solarbio, Beijing, China) in e 7500 Real-Time PCR System (Applied Biosystems, USA). Primers were listed in Table 1.

Table 1. Primer sequences.

| Gene | Forward (5′-3′) | Reverse (5′-3′) |
|---|---|---|
| USP33 | CTTGCTGCCTTCTTTGCCAG | TTTTTGTGCCTCTTCGCTGC |
| GAPDH | GGACCTGACCTGCCGTCTAG | GTAGCCCAGGATGCCCTTGA |

## Western blotting analysis

Total proteins from tissues and cells were isolated with the help of lysis buffer (Roche, Shanghai, China) supplemented with 1% protease inhibitor (Solarbio). After centrifugation at 4°C for 30 min, Bicinchoninic acid Protein Assay kits (Thermo Fisher Scientific) were applied to examine protein concentrations in light of specifications. Then, protein samples were loaded to 10% SDS-polyacrylamide gel and submitted to electrophoresis and transformation to polyvinylidene difluoride membranes (PVDF; Millipore, Billerica, MA, USA). Next, the membranes were probed with primary antibodies overnight at 4°C after being blocked in 5% nonfat milk for 1 hour at room temperature. The anti-GAPDH antibody (1:5000 dilution; cat no. ab8245, Abcam), anti-USP33 antibody (1:2000 dilution; cat no. ab237510, Abcam), anti-Bcl-2 antibody (1:2500 dilution; cat no. ab182858), anti-Bax antibody (1:2000 dilution; cat no. ab32503, Abcam), anti-Cleaved caspase-3 antibody (1:2000 dilution; cat no. ab2302, Abcam), anti-cyclinD1 antibody (1:2000 dilution; cat no. ab40754, Abcam), anti-p21 antibody (1:2000 dilution; cat no. ab109520, Abcam), anti-SP1 antibody (1:2000 dilution; cat no. #9389, Cell Signaling Technology), anti-p-AKT antibody (1:2000 dilution; cat no. #4060, Cell Signaling Technology), anti-AKT antibody (1:5000 dilution; cat no. #9272, Cell Signaling Technology) and anti-c-Jun antibody (1:4000 dilution; cat no. #9165, Cell Signaling Technology) were used in this study. Then, the membranes were probed with the HRP-conjugated secondary antibodies at room temperature for 1 hour. Following incubation with ECL reagent (Millipore, USA), the protein signaling was measured by using the ProfiBlot-48 (Tecan, Switzerland) and quantified by using ImageJ software.

## MTT assay

MTT reagent (Sigma–Aldrich) was applied for cell viability detection. In brief, RB cells were inoculated into 96-well plates ($2 \times 10^3$ cells/well) and treated with different vectors as indicated. Following incubation at 37°C for 48 hours, the cells were incubated with 20 μl MTT solution (1 mg/ml) for further 4 hours at 37°C. The MTT solution was then removed and 100 μl DMSO (Sigma–Aldrich) was added. The OD value at a wavelength of 570 nm was measured by using an automated microplate reader.

## Flow cytometry assay

Cell cycle distribution and apoptosis rates were detected by using the flow cytometry assay. Cells with different treatments were harvested and fixed in 70% ethanol overnight at -20°C. After that, the cells were incubated with 5 μg/ml of propidium iodide (PI; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in the presence of 1 mg/ml ribonuclease A (Sigma-Aldrich) for 30 min at room temperature. Cell apoptotic rates were detected using the Annexin V/PI apoptotic detection kit (BS Bioscience, San Jose, CA, USA). Cell cycle distribution and apoptotic rates were determined by using flow cytometry (BD Biosciences, San Jose, CA, USA).

## Edu staining

RB cells ($6 \times 10^3$ cells/well) were placed into 96-well plates. After 48 hours of transfection, the cells were incubated with 100 mL of EdU medium for 2 hours and fixed with 4% paraformaldehyde for 30 minutes, 0.5% Triton X-100 penetrant for 5 minutes, glycine (2 mg/mL) for 5 min at room temperature, and Apollo dye reaction liquid for 30 minutes in the dark. Hoechst 33,342 reaction in the dark for 30 min at room temperature was used for nuclear staining. Edu positive cells were proliferative cells.

## Animal experiments

The animal studies were approved by the Animal Care Committee of the Third People's

Hospital of Changzhou. Y79 cells ($2 \times 10^6$) stable transfected with shNC or shUSP33#2 were injected into the armpit of 6-week male BALB/c nude mice (5 mice in each group). Four weeks later, mice were euthanized and weighted. Tumor volume was measured every 7 days and was estimated by $V = Length \times Width^2/2$. Then, the tumors were collected for immunohistochemical staining with anti-USP33 antibody (cat no. ab237510, Abcam), anti-SP1 antibody (cat no. #9389, Cell Signaling Technology), anti-p-AKT antibody (cat no. #4060, Cell Signaling Technology) and anti-Ki67 antibody (cat no. ab15580, Abcam) at 1:100 dilution according to previously reported [23].

## Statistical analysis

SPSS22.0 (IBM Corp) was used for data analyses. Data are expressed as mean ± standard deviation (SD). The differences between the two groups and ≥3 groups were analyzed by using Student's t-test or one-way ANOVA with Tukey's tests. In detail, a paired t-test was applied for comparisons of data from RB tissues and the paired adjacent normal tissues (Figure 1a). A $p < 0.05$ was considered statistically significant.

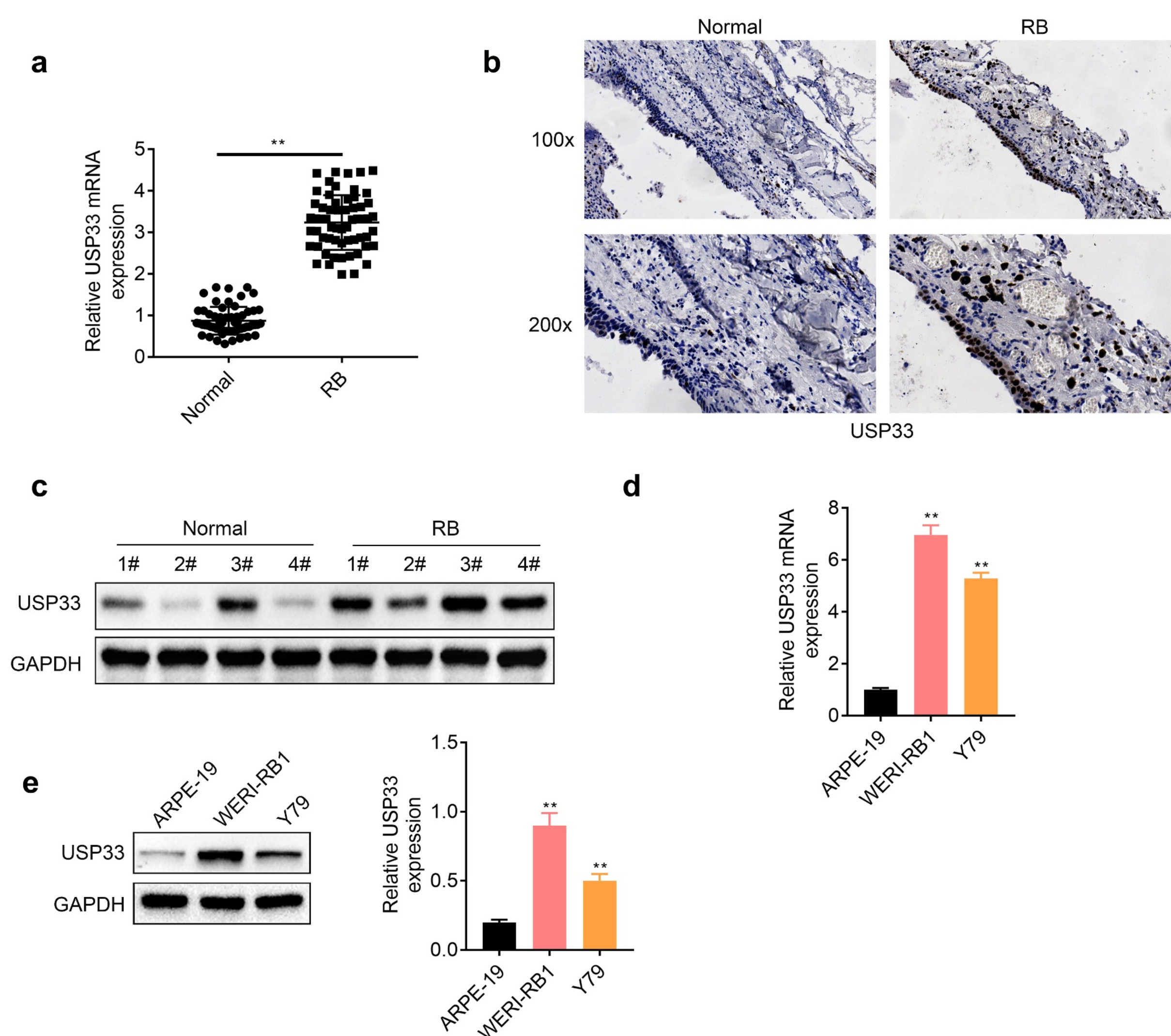


Figure 1. USP33 expression level was increased in RB tissues and cells. (a) RT-qPCR, (b) IHC and (c) western blotting assays were applied to detect USP33 levels in RB tissues and normal retinal samples. (d-e) The mRNA and protein levels of USP33 in RB cells Y79 and WERI-RB1, and retinal pigmented epithelium ARPE-19 cells were determined by RT-qPCR and western blotting. (**$p < 0.01$).

## Results

### *USP33 level was increased in RB tissue samples and cells*

To explore USP33 role in RB progression, we first determined its expression levels in RB tissues and cell lines. Compared with the adjacent normal tissues, USP33 level was significantly boosted in RB tissues, as detected by using the RT-qPCR (n = 60; Figure 1a), IHC (Figure 1b) and western blotting assays (n = 4) (Figure 1c). Additionally, USP33 level was increased in Y79 and WERI-RB1 cells, two human RB cell lines as compared with ARPE-19, the human retinal epithelial cells (Figure 1d-1e). These results demonstrated a high expression of USP33 in RB tissues and cells.

### Knockdown of USP33 repressed cell growth and induced G1 phase arrest in RB

Next, we studied USP33 role in RB progression *in vitro*. USP33 level was significantly increased following cell transfection with USP33 plasmid, and decreased when cells were infected with shUSP33#1 and shUSP33#2 as compared with the control and shNC group in both Y79 and WERI-RB1 cell lines, respectively (Figure 2a). The MTT assay result showed that USP33 overexpression increased cell viability and knockdown of USP33 impaired cell viability (Figure 2b). A similar result was observed from the Edu assay in both Y79 and WERI-RB1 cell lines (Figure 2c).

In addition, we assessed USP33 effects on cell cycle using flow cytometry and western blotting

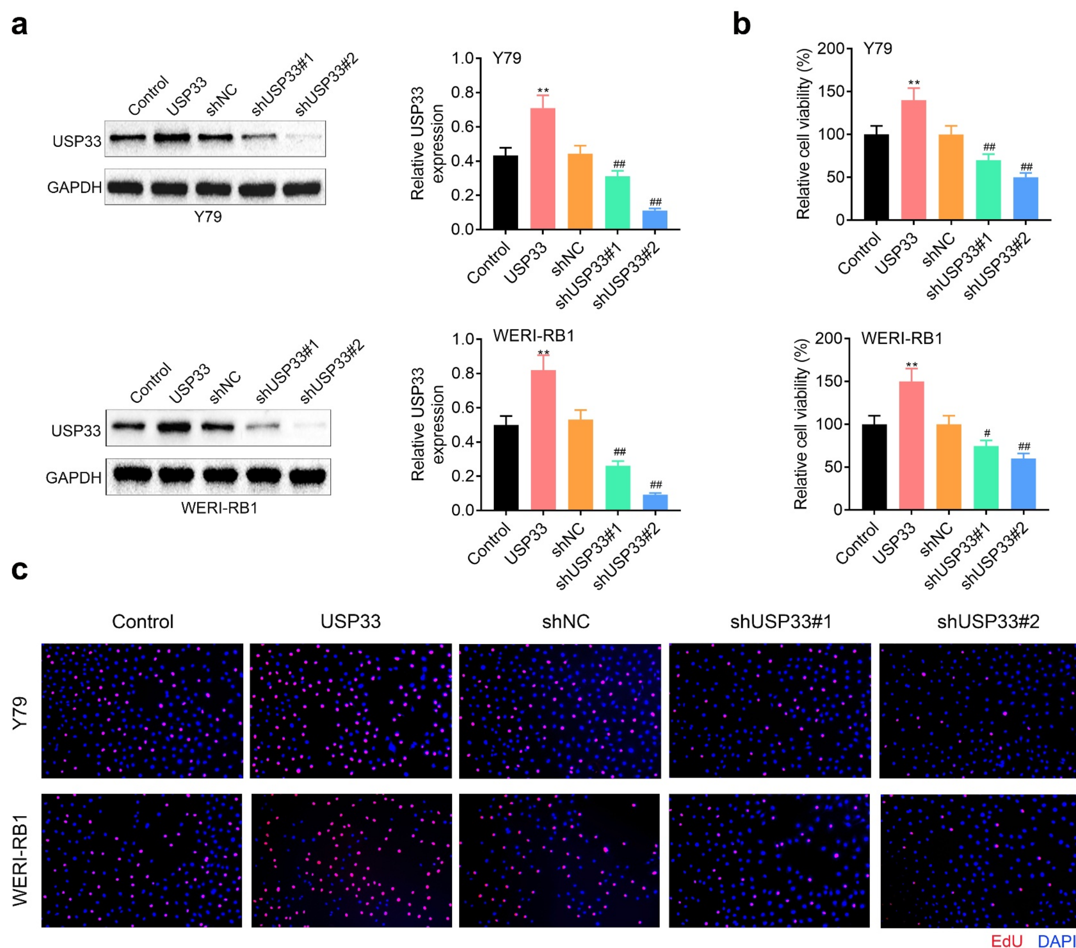


**Figure 2.** USP33 promoted RB cell growth. Y79 and WERI-RB1 cells were divided into Control, USP33, shNC, shUSP33#1 and shUSP33#2 groups, then submitted to the following assays. (a) Western blotting assay was applied to detect USP33 levels. (b-c) Cell growth was measured using MTT and Edu assays. (*$p < 0.05$, **$p < 0.01$, vs. shNC+Vector group; #$p < 0.05$, ##$p < 0.01$, vs. hUSP33#2 + Vector group).

assays. We found that USP33 upregulation induced increases in cell numbers of S and G2 phases and a reduction in G1 phase, and knock-down of USP33 caused an opposite result (Figure 3a). Compared with the control group, cell apoptotic rate was decreased when USP33 was overexpressed, and increased when USP33 was downregulated (Figure 3b). In addition, USP33 overexpression upregulated the expression levels of cyclinD1 and Bcl-2, and lowered the expression levels of p21, Bax, and Cleaved caspse-3 (Figure 3c). Taken together, the above results demonstrated that USP33 facilitated cell growth and induced apoptosis in RB cells.

## USP33 activates the PI3K/AKT/c-Jun signaling through SP1

Gan et al. [19] found that USP33 increased SP1 expression in HCC. And, SP1 can mediate the activation of PI3K/AKT/c-Jun signaling in glioma [22]. We then explored the relationship between USP33 and the SP1/PI3K/AKT/c-Jun signaling *in vitro*. Compared with the adjacent normal tissues, SP1, p-AKT, and c-Jun expression levels were all increased in RB tissues (Supplementary Figure 1). The expression levels of SP1, p-AKT/AKT, and c-Jun were significantly increased following USP33 was overexpressed in Y79 and WERI-RB1 cells, and

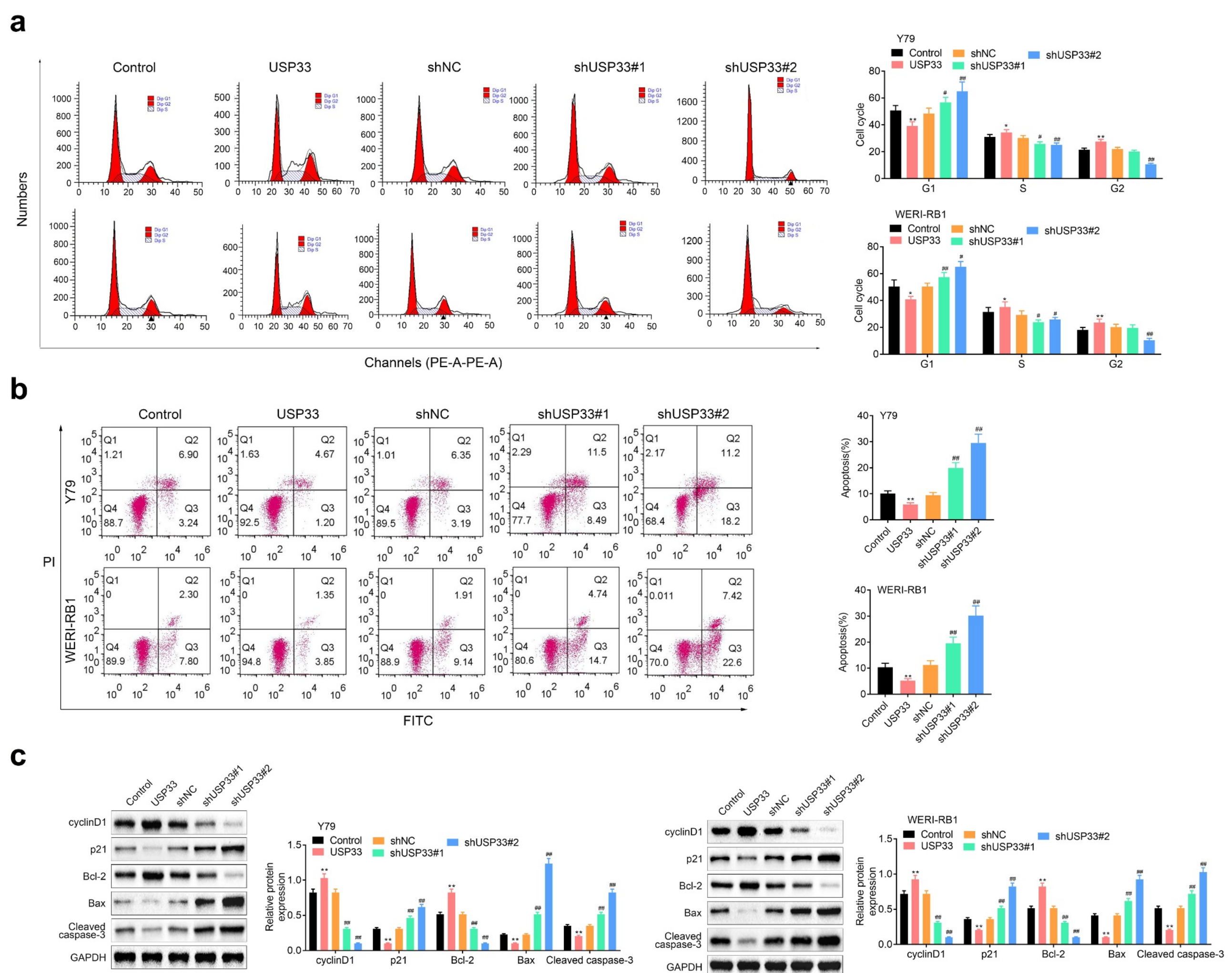


Figure 3. USP33 role in cell cycle and apoptosis. Y79 and WERI-RB1 cells were divided into control, USP33, shNC, shUSP33#1 and shUSP33#2 groups, and then the following assays were performed. (a) Cell cycle was detected by using flow cytometry assay. (b) Cell apoptotic rates were assessed by flow cytometry. (c) The expression levels of cyclinD1, p21, Bcl-2, Bax and Cleaved caspase-3 were determined using the western blotting assay. (*$p < 0.05$, **$p < 0.01$, vs. shNC+Vector group; #$p < 0.05$, ##$p < 0.01$, vs. hUSP33#2 + Vector group).

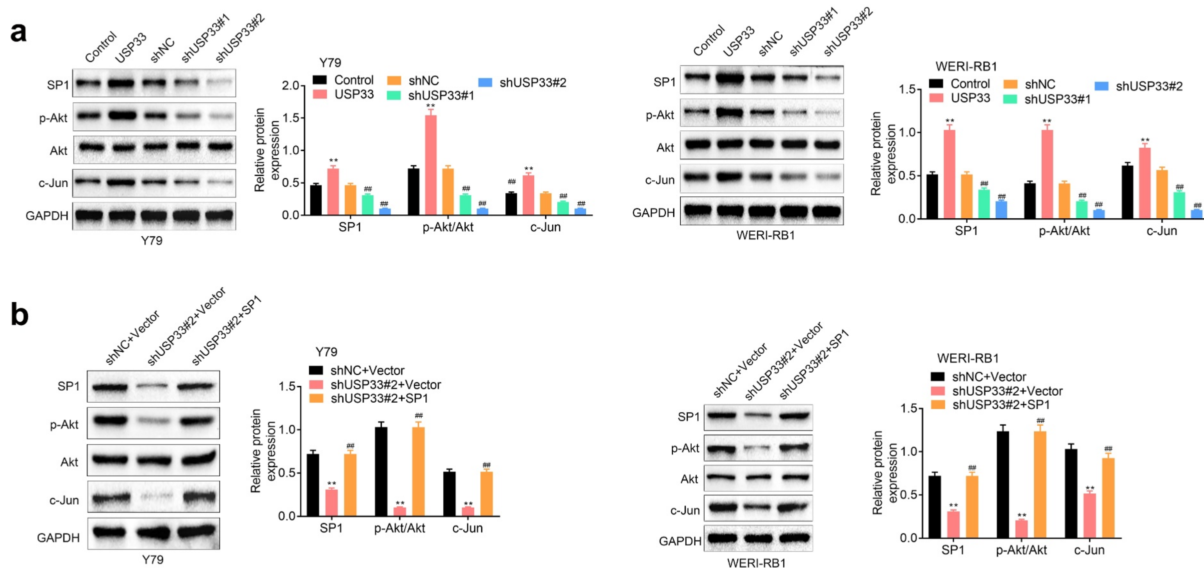


**Figure 4.** USP33 induced the activation of the PI3K/AKT/c-Jun signaling through SP1. (a-b) Western blotting assay was used to detect the protein levels of SP1, p-AKT, AKT and c-Jun in different groups of Y79 and WERI-RB1 cells. (A, $**p < 0.01$, vs. control group; $\#\#p < 0.01$, vs. shNC group; B, $*p < 0.05$, $**p < 0.01$, vs. shNC+Vector group; $\#p < 0.05$, $\#\#p < 0.01$, vs. shUSP33#2+ Vector group).

decreased when USP33 was silenced (Figure 4a). Also, we assessed whether USP33 activated the PI3K/AKT signaling in a SP1-dependent way. Overexpression of SP1 increased the levels of SP1, p-AKT/AKT, and c-Jun, which were inhibited by shUSP33#2 (Figure 4b). These results demonstrated that USP33 activated the PI3K/AKT/c-Jun signaling through SP1 in RB cells.

## Downregulation of USP33 induces G1 phase arrest and apoptosis through downregulating of SP1

Next, we explored the role of USP33/SP1 in RB cell cycle and apoptosis regulation mediated by USP33. Compared with the shUSP33#2+ Vector group, USP33 downregulation and SP1 overexpression simultaneously decreased cell number in G1 phase and induced S and G2 phase arrest (Figure 5a), inhibited cell apoptosis (Figure 5b), and increased cell viability (Figure 5c), together with increases in the expression levels of cyclinD1 and Bcl-2, and decreases in p21, Bax, and Cleaved caspase-3 (Figure 5d). These findings confirmed that downregulation of USP33 induced G1 phase arrest and cell apoptosis in RB Y79 cells through downregulating of SP1.

## Downregulation of USP33 inhibits tumor formation *in vivo*

Also, the *in vivo* assay was carried out to explore USP33 role in RB progression. Compared with the control group, tumor volume, and weight were significantly decreased when USP33 was silenced (Figure 6a). Besides, the expression levels of USP33, SP1, p-AKT, and Ki67 were significantly lower in tumors from the shUSP33 group as compared with the shNC group (Figure 6b). These results further confirmed that downregulation of USP33 inhibited tumor formation *in vivo*.

## Discussion

The current study provides evidence that USP33 exerts an oncogenic role in RB through activating SP1-mediated PI3K/AKT signaling. A higher expression pattern of USP33 was observed in RB tissues and cells as compared with normal tissues and cells. Overexpression of USP33 significantly enhanced RB Y79 and WERI-RB1 cell growth, induced a G2 and S phases arrest and inhibited cell apoptosis. To the contrary, USP33 overexpression caused marked enhancements in cell growth, G1 phase arrest and tumorigenesis and decreased

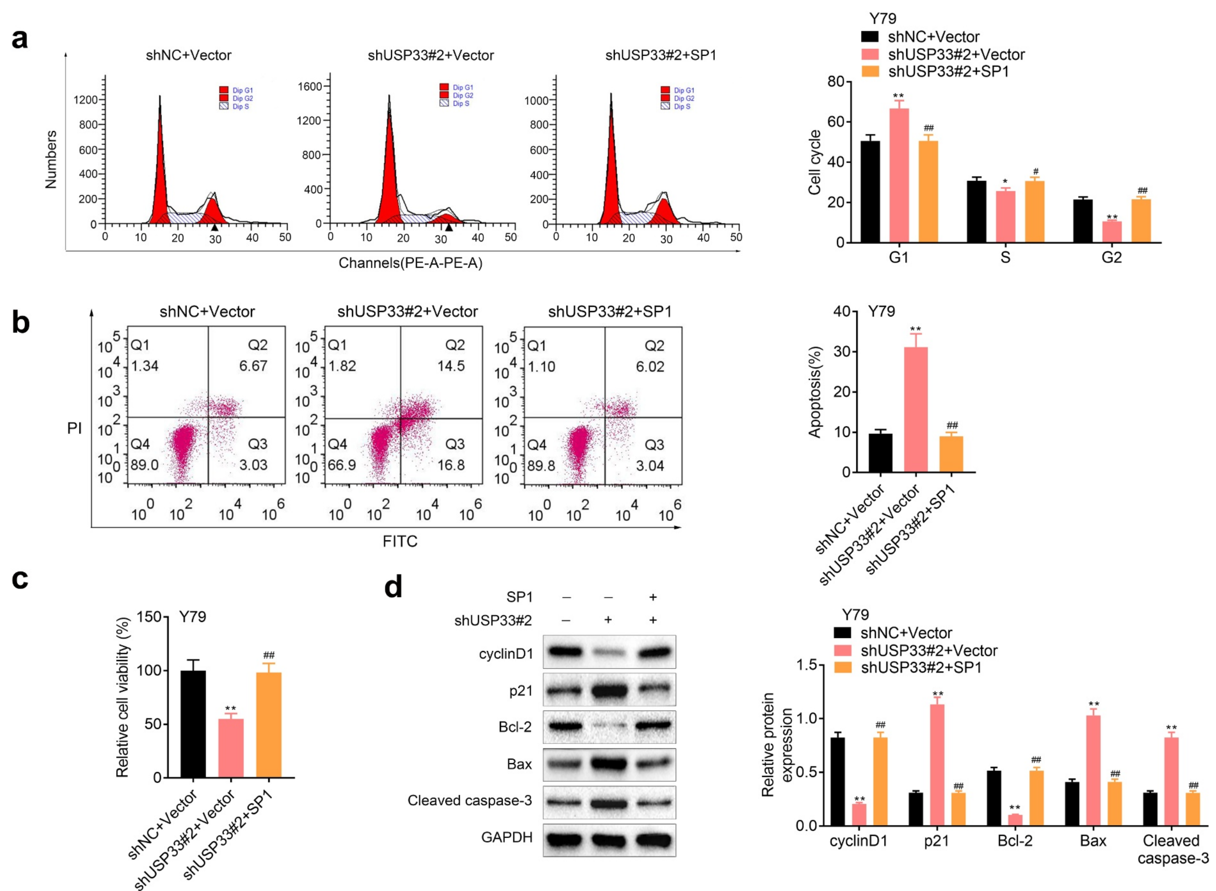


**Figure 5.** USP33/SP1 role in regulating RB cell cycle and apoptosis. Y79 cells were divided into shNC+Vector, shUSP33#2+ Vector and shUSP33#2+ SP1 groups and collected for the following detections. (a-b) Flow cytometry assay was performed to detect cell cycle and apoptosis. (c) Cell viability was detected by MTT assay. (d) Western blotting assay was used to detect the expression levels of clyclinD1, p21, Bcl-2, Bax and Cleaved caspase-3. (*$p < 0.05$, **$p < 0.01$, vs. shNC+Vector group; #$p < 0.05$, ##$p < 0.01$, vs. hUSP33#2 + Vector group).

cell apoptosis. All of the evidence demonstrated that USP33 functions as an oncogene in RB.

USP33 has been identified to be implicated in multiple cellular processes, such as cell growth, apoptosis, centrosome biogenesis [15,24,25]. Additionally, accumulated evidence has demonstrated that USP33 is strongly implicated in carcinogenesis, but is role depend on cancer contents [18,26–30]. Wen at al. [29] reported that USP33 expression was decreased in lung cancer tissue sample, which was detected in multiple microarray datasets of lung cancer and verified using RT-qPCR and IHC analysis; the lower expression level of USP33 predicted poor prognosis and mediated the Slit activity in inhibiting lung cancer cell migration. USP33 expression was shown to be decreased in colorectal cancer samples and inhibited cell migration [18]. Guo et al. [26] found that USP33 expression was elevated in prostate cancer cells and tissues, and USP33 downregulation enhanced docetaxel-induced apoptosis of prostate cancer cells, together with an increase in the phosphorylation of the cJUN NH2-terminal kinase (JNK). USP33 level was increased in HCC, and USP33 repression weakened HCC invasion and metastasis both *in vitro* and *in vivo*, indicating that USP33 acts as an oncogene in HCC [19]. These results reveal different roles of USP33 plays in various cancers, which depends on cancer contents. Here, we revealed, for the first time that USP33 was overexpressed in RB tissues and cells, which caused marked enhancements in cell growth, G1 phase arrest and tumorigenesis and decreased cell apoptosis. And, knockdown of

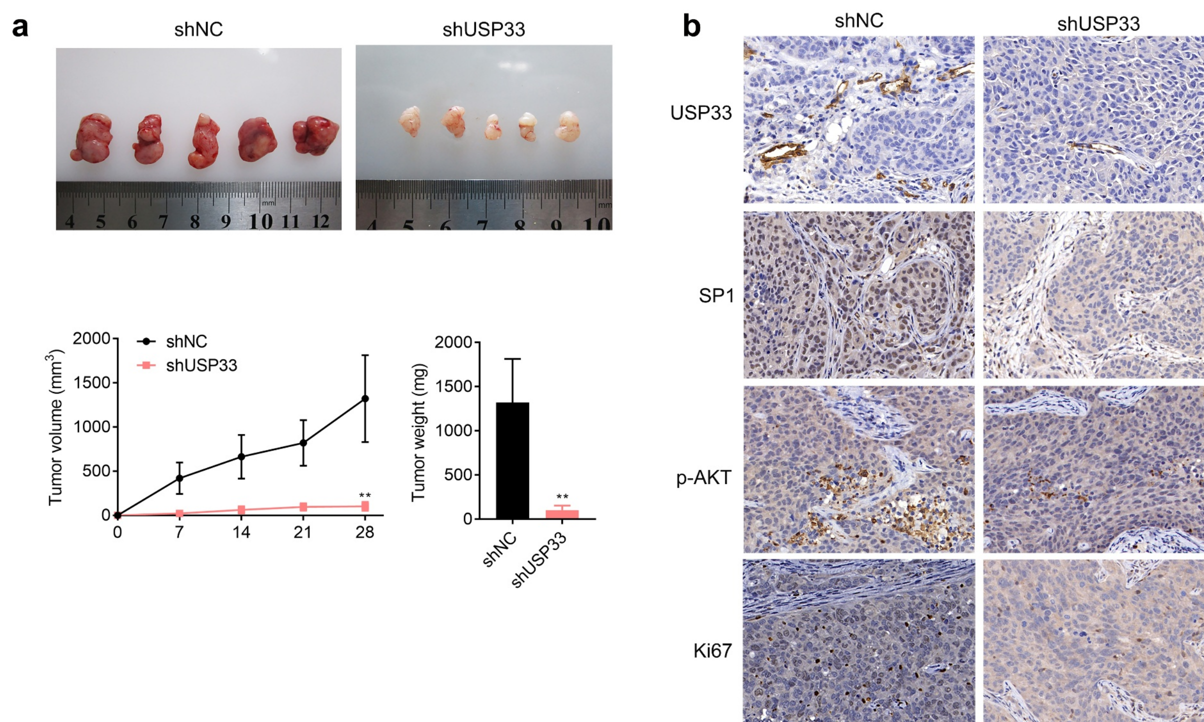


**Figure 6.** Downregulation of USP33 inhibited tumor formation *in vivo*. (a) Tumor volume and weight were assessed form mice treated with shNC or shUSP33-infected Y79 cells. (b) Immunohistochemical staining were used to determine the expression levels of USP33, SP1, p-AKT and Ki67 in mice tumor tissues. (**$p < 0.01$).

USP33 significantly enhanced RB Y79 and WERI-RB1 cell growth, induced a G2 and S phases arrest and inhibited cell apoptosis, illustrating that USP33 functions as an oncogene in RB.

SP1, as a transcription factor, promoted RB progression through increasing the expression of lncRNA PANDAR and lnc00152 [20,21]. Gan et al. [19] found that aberrant expression of USP33 increased SP1 expression through deubiquitinating of SP1 in HCC. Therefore, we assessed USP33 effect on SP1 expression in RB cells. A same result was observed as shown in HCC cells that USP33 positively modulated SP1 expression in Y79 and WERI-RB1 cells. In addition, SP1 can mediate the activation of PI3K/AKT/c-Jun signaling in glioma [22]. We also assessed USP33 role in the activation of SP1-mediated PI3K/AKT/c-Jun signaling. We observed that USP33 overexpression increased the expression levels of p-AKT/AKT and c-Jun, and USP33 downregulation decreased the expression levels of p-AKT/AKT and c-Jun, whereas the effect of USP33 downregulation was abrogated by SP1 upregulation. This result indicated that USP33 activated PI3K/AKT/c-Jun signaling through increasing SP1 expression in RB.

Moreover, we also assessed the role of USP33/SP1 role in RB progression *in vitro*. As expected, SP1 overexpression impaired USP33 downregulation-mediated inhibition in cell growth and promotion in cell apoptosis. Also, the animal assay showed that downregulation of USP33 inhibited tumor growth, accompanied by decreases in the expression levels of SP1 and p-AKT. These results confirmed that USP33 promoted RB progression in a SP1-dependent manner.

USP33 is a deubiquitinating enzyme and takes part in multiple kinds of disease through modulation of the ubiquitin degradation pathway. For instance, Wen et al. [29] demonstrated that USP33 stabilized Robo1 protein by reducing proteasome-dependent degradation. Also, USP33 endowed the inhibitory role of Slit2 on colorectal cancer cell migration by deubiquitinating and stabilizing Robo1 [18]. However, we didn't reveal the substrates, which is deubiquitinated by USP33 and then accelerate RB progression.

## Conclusion

This study uncovered that USP33 promoted the progression of RB through regulation of the SP1/PI3K/AKT pathway. This paper would provide a therapeutic suggestion, such as proteasome inhibitors might be hopeful reagents for RB treatment. However, more trials are needed to be done to move this into the clinic.

## Acknowledgments

Not applicable.

## Disclosure statement

No potential conflict of interest was reported by the author(s).

## Funding

The authors have no funding to report.

## Ethics approval

All procedures performed in studies involving human participants were in accordance with the standards upheld by the Ethics Committee of the Third People's Hospital of Changzhou and with those of the 1964 Helsinki Declaration and its later amendments for ethical research involving human subjects.

All animal experiments were approved by the Ethics Committee of the Third People's Hospital of Changzhou for the use of animals and conducted in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines.

## Statement of Informed Consent

Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.

## Availability of data and materials

All data generated or analyzed during this study are included in this published article.

## Authors' contributions

Hao Wang and Zhinan Liu designed the study, supervised the data collection, Zhuo Sun analyzed the data, interpreted the data, Dong Zhou, Hanyan Mao, and Guohua Deng prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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